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(71) Applicant *(for all designated States except US)*: MEDVET SCIENCE PTY LTD. [AU/AU]; IMVS Building, Level 3 South Wing, Frome Road, Adelaide, S.A. 5000 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FINDLAY, David [AU/AU]; (AU). FAZZALARI, Nicola [AU/AU]; (AU). KULIWABA, Julia [AU/AU]; IMVS Building, Frome Road, Adelaide, S.A. 5000 (AU). FORWOOD, Mark [AU/AU]; University of Queensland, Dept. of Anatomical Science, St Lucia, QLD 4072 (AU).

(74) Agent: A.P.T. PATENT AND TRADE MARK ATTORNEYS; G.P.O. Box 772, Adelaide, S.A. 5001 (AU).

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(54) Title: PREDICTIVE ASSESSMENT OF CERTAIN SKELETAL DISORDERS

(57) Abstract

A method of predicting or diagnosing a skeletal disorder in an individual. The method including the steps of taking a sample of body tissue or body fluid, measuring or estimating the level of at least one regulator or marker of bone remodelling in the sample, and comparing the level to a standard to determine whether the level of the regulator or marker falls within a range indicative of a potential of the individual to progress to exhibit overt symptoms of the skeletal disorder. Suitable markers or regulators include IL–1α, IL–1β, IL–6 and its soluble and cell membrane receptor, IL–11 and its receptor, IL–17 and its receptor, M–CSF, ODF, RANK, TRAIL and CTR, IL–18, OPG, BMP2, BMP6, BMP7, TGFβ1, TGFβ2, TGFβ3, IGFI, alkaline phosphatases, OCN, and fragments of type I collagen.

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PREDICTIVE ASSESSMENT OF CERTAIN SKELETAL DISORDERS

FIELD OF THE INVENTION

This invention relates to a predictive assessment of certain skeletal disorders.

BACKGROUND OF THE INVENTION

Osteoarthritis (OA) and osteoporosis (OP) are two common conditions of the skeletal system which both occur with greater frequency in advanced age. These conditions can become quite crippling and remedial surgery is often needed in severe cases to alleviate pain and to enhance mobility of the affected individual.

In the case of osteoarthritis the progression of the disease has been described by Collins (1949) in terms of four diagnostic grades on the basis of macroscopic examination of joint surfaces, termed I, II, III and IV, respectively, reflecting increasing severity. Typically grades III and particular IV are considered severe grades with the milder forms of osteoarthritis that fall into grades I and II often having minimal impact on quality of life.

Generally treatment of osteoarthritis is that of management and relief of symptoms. It is considered possible to limit the progression of the condition rather than being able to facilitate a regression of the condition. Thus anti-inflammatory drug treatment can slow the progression of OA, with severe cases of OA the approach to management is to provide palliative care but with more advanced cases surgical intervention such as arthroplasty is required. In the case of OP, exercise, hormone replacement therapy and maintenance of dietary calcium levels can influence the maintenance of bone mass and prevent fractures.

It would be highly desirable to be able to establish a predictive assay whereby the predisposition of individuals to a skeletal disorder such as osteoarthritis or osteoporosis can give an indication of the probability of an individual progressing to a severe case, which could enable individuals to take steps to delay onset of disease and/or targeting of individuals for treatment. Thus, for example, exercise is promoted as being good for general health but can actually exacerbate or accelerate the onset of severe OA.

Osteoarthritis is usually considered to be a disorder of the joint cartilage, with secondary changes in the underlying subchondral bone. There is growing evidence, however, that the origins of osteoarthritis reside in bone. If the origins of OA do reside in bone, structural changes in the subchondral bone that result in reduced shock absorption capability would result in altered load bearing properties. Thus, the load on the joint would be borne to a greater extent by the articular cartilage, resulting in gradual degeneration of the cartilage (Dequeker *et al.*, 1993; Dequeker *et al.*, 1997). This is in contrast to the more commonly held view that the reverse is true, that is, the degenerative articulate cartilage results in bone pathology.

Supporting the above view, bone from the iliac crest of primary OA patients, being distant from the affected joints, has been shown to have increased stiffness, compressive strength, osteocalcin content, associated with increased content of insulin-like growth factor types I and II (IGF-I and IGF-II) and transforming growth factor β (TGF-β), compared with that in control subjects (Dequeker *et al.*, 1993; Dequeker *et al.*, 1993a). The quality of OA bone at a distance from the site of joint deterioration suggests that individuals who will develop OA may be predisposed to develop bone that is stiffer than normal as a result of a remodelling imbalance that favours bone formation (Dequeker *et al*, 1997). OA has been found to have a low incidence of osteoporotic hip fracture in both men and women (Dequeker *et al.*, 1993). The maintenance of bone volume in ageing OA individuals may be protective against hip fracture (Dequeker *et al.*, 1995). Therefore, it might be expected that factors involved in the bone remodelling process would exhibit a different mRNA expression pattern in OA compared with control bone, even at a site distant to the affected joint.

Understanding of the pathogenesis of OA and OP is still at the very early stages. A large amount of *in vitro* experimental work has been undertaken to investigate the growth factors and cytokines that regulate the differentiation and function of bone cells. However, little is known about the *in vivo* expression of these mediators and their receptors in the bone microenvironment in humans and whether expression is altered in disease. The cytokines interleukin-6 (IL-6) and interleukin-11 (IL-11) are both involved in osteoclastogenesis. IL-11 has been postulated to have an important role in the regulation of osteoclastogenesis since neutralisation of IL-11 suppressed osteoclast development induced by a number of pro-resorptive agents;

1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), parathyroid hormone (PTH), interleukin-1 (IL-1), or tumour necrosis factor (TNF) (Girasole *et al.*, 1994; Manolagas, 1995). IL-6 possesses biological functions similar to IL-11 and shares the common β subunit receptor, gpl30, in signal transduction (Kishimoto *et al.*, 1992). Unlike IL-11, IL-6 itself may not promote osteoclast formation *in vivo* under physiological conditions, even though it has been shown to stimulate bone resorption by enhancing the formation and activity of osteoclasts (Tamura *et al.*, 1993). Shanbhag *et al.* (1995) cultured synovial tissue cells from patients with OA and autopsy control individuals, and found that control tissue secreted significantly higher levels of IL-6 ($p < 0.01$). In addition to the role IL-11 has in osteoclastogenesis, IL-11 has been implicated as a suppressor of osteoblastic activity. This was shown by an IL-11 dose dependent inhibition of nodule formation and reduction in alkaline phosphatase expression in rat calvaria cell cultures (Hughes and Howells, 1993). Gevers and Dequeker, (1987) and Dequeker *et al.* (1993) found an increased osteocalcin (OCN) in iliac crest bone and postulated that there may be an increased biosynthetic activity of osteoblasts in OA.

None of the above work has been of a predictive nature or suggested a manner in which the OA or OP status of individuals could be predicted.

SUMMARY OF THE INVENTION

The present invention arises from the finding that the level of expression of any one or more of a number of regulators or markers of bone remodelling varies in osteoarthritic bone. The invention is predicated on the finding that differences in expression of the regulators or markers are age related and therefore the level of any one or more of such regulators or markers either on its own or more preferably in combination with others can be used as a predictive measure of the potential for onset of a severe form of certain skeletal disorders.

Thus in a first aspect the invention might be said to reside in a method of predicting or diagnosing a skeletal disorder in an individual, said method including the steps of taking a sample of body tissue or body fluid and measuring or estimating the level of at least one regulator or marker of bone remodelling in the sample. Typically the level will be compared to a standard to determine whether the level of the regulator or marker falls within a range indicative of a potential of the individual to progress to

exhibit overt symptoms of the skeletal disorder. The standard may be ascertained by referring to a table or graph or alternatively it might be compared to an experimental control.

The body tissue sampled may be bone or any other tissue in which regulators of markers or bone remodelling are found. In the case of a bone sample the method may involve estimating the levels of marker or regulator in cells isolated from regions other than the subchondral region of the bone. The method may include the extraction of mRNA from cells in bone tissue and estimating the level of expression for the one or each of the regulators of bone remodelling by measuring the quantity of mRNA specific for that regulator.

Alternatively the body fluid sampled may be blood, urine, or any other body fluid in which regulators of markers or bone remodelling are found.

The regulator of bone remodelling used for the present invention may have a narrow range of activity in the body associated with bone remodelling and might be a localised bone autocrine or paracrine regulator or associated protein or receptor or might be a more general regulatory compound that has an effect on bone remodelling, such as a growth factor. The regulator will be an intrinsic compound, expression of which might alter bone formation or bone resorption. Many of the regulators known to exert a regulatory effect on bone remodelling are cytokines and associated proteins such as receptors, or generalised factors such as insulin growth factors. Thus the regulator or marker thereof might be selected from the group of regulators or markers associated with bone resorption comprising IL-1α, IL-1β, IL-6 and its soluble and cell membrane receptor, IL-11 and its receptor, IL-17 and its receptor, M-CSF, ODF, RANK, TRAIL and CTR, or the group of inhibitors of bone resorption including IL-18, OPG or the group of regulators or markers associated with bone formation comprising BMP2, BMP6, BMP7, TGFβ1, TGFβ2, TGFβ3, IGFI, alkaline phosphatases, OCN, and fragments of type I collagen. It will be understood however that this list cannot be considered exhaustive because regulators of bone remodelling are still being discovered.

In one form of the first aspect of the invention the level of more than one regulator or marker of bone remodelling is estimated and an assessment is made on the basis of

the combination of more than one score. It is anticipated that more than one regulator or marker will need to be measured, because the regulators or markers of bone remodelling assessed to date will, taken alone, all lead to a proportion of false positives or negatives, and combinations of more than one are anticipated to reduce the proportion of false positives or negatives.

An unexpectedly low deviation from the mean for the measure of the ratio of a regulator enhancing bone resorption or marker thereof compared to a regulator enhancing bone formation or marker thereof has been found in individuals with OA. Accordingly this is a measure that provides a good predicability. Ratios of pairs of resorption and formation regulators may give a similar result, or composite ratios of these may be used. Alternatively ratios of values corresponding to any two or more regulators or markers might be of benefit in the predictive assay. For example the ratio of the markers CTR/OCN mRNA was shown to be a strong indicator of OA.

A number of methods might be used to assess the level of the regulator or marker. Thus it is known that bone turnover may be reflected by the concentration in serum and urine of either enzymatic activities of the bone cells involved in the turnover processes or by-products which are released during bone formation or bone resorption. Markers of bone formation or resorption that may be assayed according to the invention include alkaline phosphatase (Chisaka, 1991; Miric *et. al.*, 1992), OCN (Price *et. al.*, 1981), and fragments of type I collagen whose levels correlate with bone formation and/or resorption (Prockop *et. al.*, 1979). Thus measurement of any one or more of these markers in serum or urine and comparison to a control group may provide predicability of the onset of OA.

Alternatively the quantity of target molecules might be measured directly by, for example, detection using monoclonal antibodies to the regulators or markers concerned. However, in one preferred form of the invention the method includes the extraction of mRNA from a bone sample and estimating the level of expression for the one or each of the regulators of bone remodelling by measuring the quantity of mRNA specific for that regulator. The measurement has been made using an extraction of total RNA from human bone tissue obtained at joint replacement surgery or at autopsy. The latter to our knowledge, has not been reported before. Marchuk *et al.* (1998) showed the stability of total RNA extracted from rabbit

ligament, tendon, and cartilage tissue obtained up to 96 hours postmortem. We found no evidence for any significant degradation of IL-6, IL-11, CTR, OCN, OPG, ODF or RANK mRNA up to 120 hours postmortem. The use of mRNA enables convenient measurement of a number of parameters with the same sample, and using similar reagents, simply by altering the probes used, expression of different growth factors or regulatory factors can readily be quantitated.

The source of the bone sample might be a biopsy sample such as from the iliac crest. Measurement of activity is preferably from cells isolated from cancellous bone. It is anticipated, in the form of the invention where biopsy is taken, that the test is not envisaged as being a general screening test but would be conducted for individuals considered at risk of manifesting severe symptoms of the skeletal disorder. Thus for OA, individuals at risk would be those with affected family members, or known to have sustained joint damage at some time. A proportion of these individuals will go on to develop severe OA in a joint and presumably this proportion will be greater in predisposed individuals. Alternatively X rays, CT or MRI of the joint, that show signs of OA changes, could be used to identify individuals for bone biopsy.

The data to hand is for OA however the skeletal disorder that is predicted might not only be osteoarthritis. Based on a view which we believe to be accurate, OA is a bone disorder whereby the bone balance of remodelling is skewed in the favour of increasing bone, and OP is a bone disorder whereby the bone balance of remodelling is skewed in the favour of bone loss. Thus another skeletal disorder amenable to prediction is OP. There may also be other diseases that are caused by disturbance of the bone balance that is characteristic of normal bone remodelling.

This invention is predicated on the assumption that there are individuals susceptible to OA with a long term tendency to express a particular profile of regulators or makers of bone remodelling and there is a very strong chance that this reflects a genetic predisposition.

It will be understood that the invention has a primary application for humans, however the invention may also have application for other mammals where these skeletal conditions are observed, such mammals might include domestic, sporting, or

agricultural animals. It is well known, for example, that race horses commonly sustain OA with a predisposition believed to have a genetic component.

For a better understanding the invention will now be described with reference to a number of examples which are explained with reference to a number of drawings wherein,

List of abbreviations used throughout the specification

IL : interleukin
M-CSF : macropahge colony stimulating factor
ODF : osteoclast differentiating factor
RANK : receptor-activator of NF-KB
RANKL : receptor-activator of NF-KB ligand
TRANCE : TNF related activation induced cytokine
OPG : osteoprotegerin
OPGL : osteoprotegerin ligand
SOFA : stromal osteoclast forming activity
OCIF : osteoclastogenesis inhibitory factor
TR1 : TNF receptor like (1)
BMP : bone morphogenetic protein
TGFß: transforming growth factor ß
IGF-I : insulin-like growth factor
CTR : calcitonin receptor
OCN : osteocalcin

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a typical pattern of mRNA expression of IL-6, IL-11, CTR, OCN and GADPH for OA and control cancellous bone samples from the proximal femur,

Figure 2a shows the levels of IL-6 and IL-11 mRNA expression in OA and control cancellous bone samples from the proximal femur,

Figure 2b shows the levels of CTR and OCN mRNA expression in OA and control cancellous bone samples from the proximal femur,

Figure 3 is a plot of CTR versus OCN mRNA expression and shows a significant positive correlation in the OA cancellous bone sample from the proximal femur; the control group does not correlate,

Figure 4 is a plot of the ratio of CTR/OCN mRNA expression in OA and control cancellous bone samples from the proximal femur,

Figure 5a is a plot showing the dependence of IL-11 mRNA expression on age for OA and control cancellous bone samples from the proximal femur,

Figure 5b is a plot showing the dependence of CTR mRNA expression on age for OA and control cancellous bone samples from the proximal femur,

Figure 5c is a plot showing the dependence of OCN mRNA expression on age for OA and control cancellous bone samples from the proximal femur,

Figure 6 is a plot of the dependence of CTR/OCN ratio on age for OA and control cancellous bone samples from the proximal femur,

Figure 7 is a plot of the ratio of ODF/GAPDH mRNA expression in OA and control cancellous bone samples,

Figure 8 is a plot of the ratio of RANK/GAPDH mRNA expression in OA and control cancellous bone samples,

Figure 9 is a plot of the ratio of ODF/OPG mRNA expression in OA and control cancellous bone samples,

Figure 10 is a plot showing the dependence of RANK mRNA expression on age for OA and control cancellous bone samples,

Figure 11 is a plot showing the dependence of ODF mRNA expression on age for OA and control cancellous bone samples.

DETAILED DESCRIPTION OF THE INVENTION

EXAMPLE 1 - *Extraction and measurement of mRNA levels from bone tissue*

Material and Methods

Human bone specimens

Proximal femur surgical specimens were obtained from 16 patients (8 from left side and 8 right) undergoing total hip arthroplasty for primary OA (8 women, 49-84 years, and 8 men, 50-85 years; mean ± SD age 68.6 ± 11.8 years). The control group (12 left and 2 right) was selected from routine autopsy cases (7 women, 20-83 years, and 7 men, 24-85 years; 61.6 ± 29.6 years), known not to have suffered from any disease affecting the skeleton. Informed consent was obtained for the collection of these specimens; with approval by the Royal Adelaide Hospital ethics committee. The mean age of the OA group did not significantly differ from the control group. Clinical details, including cause of death, height, weight, and days bed rest were obtained for the control group. Cancellous bone samples were taken for RNA extraction. A 10 mm tube saw core biopsy from the intertrochanteric region of the femur was taken for each osteoarthritic case at surgery; and a cancellous bone sample was cut, with a band saw, from the same site for the control group. This site was chosen because the trabecular architecture in this region depends upon stresses in the proximal femoral shaft while being unaffected by the sclerotic and cystic changes of the osteoarthritic femoral head (Fazzalari *et al.*, 1998).

RNA extraction

The fresh surgical cancellous bone (after storage at 4°C up to 12 hours) and control bone (obtained 2-4 days after death) was washed briefly in diethylpyrocarbonate (DEPC)-treated water (ICN Biomedicals Inc., Aurora, Ohio, USA), and then separated into small fragments using bone cutters. Total RNA was extracted from bone tissue using a modification of the guanidinium thiocyanate-phenol chloroform

protocol described by Chomzynski and Sacchi (1987). Briefly, bone fragments were placed in 4M guanidinium thiocyanate solution (10 ml per 2 g wet weight) and homogenised using an Ultra- Turrax (TP 18-10; Janke & Kunkel, KG, IKA WERK, Staufen i. Breisgau, Germany). The homogenised sample was centrifuged at low speed (1000 x g, 5 minutes) to remove any insoluble material. A 0.1 volume of 2M sodium acetate, pH 4.0, was added to the cleared homogenate, vortexed, and the mixture extracted with phenol/chloroform/isoamylalcohol. Total RNA was isopropanol precipitated and re-extracted with a 0.1 volume of 3M sodium acetate, pH 5.2, and phenol; then with chloroform/isoamyalcohol. To remove contaminating proteoglycans, 3 volumes of 4M sodium acetate, pH 7.0, was added to the aqueous phase, and precipitated at -20°C overnight. Total RNA was recovered by centrifugation, washed with 75% ethanol, and air dried. RNA was dissolved in an appropriate volume of DEPC-treated water, and stored at -70°C until further use. Total RNA was also isolated from cultured human osteoblasts obtained by out growth from trabecular chips, which were treated for 48 hrs with 1,25-dihydroxyvitamin D_3 (10^{-8} M), and MCF-7 human breast cancer cells to provide positive controls for amplification of IL-6, IL-11, OCN, CTR, OPG and ODF mRNA. The positive control for RANK mRNA was RNA extracted from giant cell tumours of bone. RNA purity and integrity was confirmed on ethidium bromide stained 1% wt/vol agarose-formaldehyde gels prior to use for RT-PCR. Sample concentration and the 260/280 ratio were determined by spectrophotometry. Total RNA isolated by this method consistently yielded 260/280 ratios of 1.7 - 2.0.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

First strand complementary DNA (cDNA) was synthesized from 1.6 µg of total RNA from each sample. The 20 µl transcription mixture contained 24 U of avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI, USA), 0.8 µg random primers (Promega), 10 mM dATP, dGTP, dCTP, and dTTP (Pharmacia Biotech, Uppsala, Sweden), and 32 U of RNasin (Promega). After initial denaturation of the RNA at 70°C, the transcription reaction was allowed to proceed at 30°C for 10 minutes, 42°C for 45 minutes, and then terminated at 95°C for 5 minutes. From this reaction mixture 1 µl of cDNA was amplified by PCR to generate products corresponding to mRNA encoding human IL-6, IL- 11, OCN, CTR, OPG, ODF, RANK and the housekeeping gene GAPDH. The 20 µl amplification mixture contained 1.25 U of Ampli*Taq* Gold DNA polymerase (Perkin Elmer, Norwalk, CT,

USA), 100 ng each of the 5' and 3' primers, 2 mM dATP, dGTP, dCTP, and dTTP (Pharmacia Biotech, Uppsala, Sweden), 1.5 mM $MgCl_2$, 2 µl 10X reaction buffer, and sterile DEPC-water. PCR, using human specific primers, was performed for 30-35 cycles for IL-6, IL-11, CTR ODF, OPG, and RANK, 30 cycles for OCN, and 26 cycles for GAPDH in a DNA thermal cycler (Corbett Research, Melbourne, Vic, Australia). After an initial step at 95°C for 9 minutes to activate the polymerase; each cycle consisted of 1 minute of denaturation at 94°C, 1 minute of annealing at 62°C for IL-6, IL-11, OCN, OPG, ODF and RANK or at 60°C for CTR, GAPDH, and 1 minute of extension at 72°C. This was followed by an additional extension step at 72°C for 5 minutes. Human specific oligonucleotide primers were designed on the basis of published sequences for IL-6, IL-11, OCN, CTR, OPG, ODF, RANK and GAPDH (Life Technologies, Gaithersburg, MD, USA). In all cases, the primers were RNA specific in that the recognition sites of the upstream and downstream primers resided in separate exons or at intron/exon boundaries in the genomic sequence. Primer sequences and predicted PCR product sizes are shown in Table 1. Amplification of GAPDH served as an internal positive control for the integrity of the extraction/reverse transcription and PCR processes and was positive in all patients studied. To allow semiquantitation of the PCR products, preliminary experiments were performed to ensure that the number of PCR cycles were within the exponential phase of the amplification curve. Amplification products were resolved by electrophoresis on a 2% wt/vol agarose gel post stained with SYBR-1 Green (Cat. No. S-7565; Molecular Probes, Eugene, OR, USA). The intensity of the PCR products was quantified using a FluorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). IL-6, IL-11, OCN, and CTR are represented as a ratio of PCR product/GAPDH. To show that there were no false-positive results, PCR reactions were carried out on non-reverse transcribed RNA, and on samples in which no RNA was added to the reaction mixture. The specificity of the PCR reaction was confirmed by Southern transfer onto a nylon membrane (Hybond-N; Amersham Life Science Inc., Arlington Heights, IL, USA) and hybridisation with a digoxigenin (DIG)-labelled internal oligonucleotide probe. To authenticate our autopsy total RNA extraction, we obtained records of the number of hours post-death before the collection of samples (ranging from 24 to 120 hours), and for each of the mRNA species there was no evidence for any significant degradation with time.

Data analysis

RT-PCR was performed in duplicate for several samples, confirming that repeated RT-PCR analysis of the same samples yielded reproducible results. The statistical significance of the difference between the control and the experimental group was determined by Student's t-test (EXCEL, Microsoft Corp., USA). Regression analysis was used to examine the relationship between PCR products. The critical value for significance was chosen for P = 0.05.

Results

RT-PCR was employed to examine the expression of IL-6, IL-11, CTR, OCN, OPG, ODF and RANK mRNA in cancellous bone sampled from the intertrochanteric region of the proximal femur for OA patients and autopsy control individuals. Total RNA was isolated from these fresh bone specimens, reverse transcribed, and cDNA was then PCR amplified using human specific primer pairs. The typical pattern of mRNA expression for IL-6, IL-11, CTR, and OCN for the OA and control groups is shown in Figure 1. The number of PCR cycles employed for each of these transcripts was within the exponential phase of the amplification curve. This allowed the semi-quantitation of IL- 6, IL-11, CTR, OCN, OPG, ODF and RANK mRNA by normalising these values to GAPDH mRNA.

Female and male IL-6, IL-11, CTR, and OCN mRNA expression

The pooled OA and control group data showed no difference between females and males for IL- 6/GAPDH (female, 0.17 ± 0.13 [mean ± SD], n = 15; male, 0.14 ± 0.06, n = 15; p = NS (not significant]), IL-11/GAPDH (female, 0.20 ± 0.12; male, 0.13 ± 0.06; p = NS), CTR/GAPDH (female, 0.56 ± 0.29; male, 0.94 ± 0.75, p = NS), and OCN/GAPDH (female, 0.90 ± 0.66; male, 0.75 ± 0.56; p = NS) mRNA expression. When the data was separated into the OA and control groups (Table 2), the only significant differences found were reduced IL-11/GAPDH mRNA expression in OA males (female, 0.12 ± 0.04; male, 0.09 ± 0.02; $p < 0.05$), and elevated CTR/GAPDH mRNA expression in control males (female, 0.46 ± 0.23; male, 1.39 ± 0. 88, $p < 0.05$).

Elevated OCN mRNA expression is associated with a reduction in IL-6 and IL-11 mRNA in osteoarthritis

There was a distinct difference in the range of IL-6, IL-11, CTR, and OCN mRNA expression between the OA and control groups (Figure 2). In OA the mRNA

expression of IL-6/GAPDH (OA, 0.12 ± 0.06, *n* = 16; control, 0.19 ± 0.12, *n* = 14; p < 0.05) and IL-11/GAPDH (OA, 0.11 ± 0.04; control, 0.23 ± 0.10; p < 0.001) were significantly reduced in comparison to the control group (Figure 2A). Whereas OCN/GAPDH mRNA expression was significantly elevated in OA (OA, 1.25 ± 0.54; control, 0.34 ± 0.10; p < 0.001) (Figure 2B). Expression of CTR/GAPDH mRNA tended to be lower in OA than in the control group (OA, 0.60 ± 0.30; control, 0.92 ± 0.78; p = NS); but this was not significant (Figure 2B).

The association of IL-6 to IL-11, and CTR to OCN

IL-6 and IL-11 share several biological activities and have been shown to have important roles in osteoclast development (Manolagas and Jilka, 1995). The functional redundancy of these cytokines can be explained by the common signal transducer, gpl30, which forms receptor complexes with specific receptors for each cytokine (Kishimoto *et al.*, 1994). A significant positive association was observed between IL-6/GAPDH and IL-11/GAPDH mRNA expression (r = +0.56, p < 0.001) in the pooled data sample (not shown); which may imply a coordinated expression of these two cytokines in the bone microenvironment. When this IL-6/GAPDH to IL-11/GAPDH association was examined independently in the two groups, only the control group was found to be significant (OA, r = +0.16, p = NS; control, r = +0.49, p < 0.05). The CTR and OCN were selected as representative markers of osteoclasts (resorption) and osteoblasts (formation). Thus, the plot of CTR/GAPDH against OCN/GAPDH mRNA expression is probably indicative of the balance of resorption to formation in these two study groups. There was a positive association between CTR/GAPDH and OCN/GAPDH mRNA expression in the OA group (r = +0.52, p < 0.05) (Figure 3). Whereas the control group showed no association between CTR/GAPDH and OCN/GAPDH (r = -0.32, p = NS). The statistical variance in the IL-6/IL-11 (OA, 1.14 ± 0.64; control, 0.86 ± 0.39; p = NS) and CTR/OCN (OA, 0.51 ± 0.21; control, 3.14 ± 3.26; p < 0.01) ratios was calculated and expressed as the ratio of control to OA. This showed that the variance of the control group was significantly increased for the CTR/OCN ratio (15.27, p < 0.001) (Figure 4); and that there was no difference in variance for the IL-6/IL-11 ratio (1.62, NS).

EXAMPLE 2 - *Associations of IL-6, IL-11, CTR, OCN, OPG, ODF and RANK mRNA expression with age*

IL-11/GAPDH (OA, r = -0.50, $p < 0.05$; control, r = -0.60, $p < 0.05$) mRNA expression declined with age in both the OA and control groups (Figure 5A). In contrast, there was a positive association of CTR/GAPDH mRNA expression with age (OA, r = +0.60, $p < 0.01$; control, r = +0.22, p = NS), which was only significant in the OA group (Figure 5B). OCN/GAPDH mRNA expression increased significantly with age in OA (r = +0.63, $p < 0.01$); whereas the control group showed a decline with age (r = - 0.63, $p < 0.01$) (Figure 5C). IL-6/GAPDH mRNA expression showed no dependence on age (OA, r = -0.24, p = NS; control, r = -0.38, p = NS). Significant associations have been found for IL-11/GAPDH, CTR/GAPDH, and OCN/GAPDH mRNA expression with age for OA and the control group; and relationships between IL-6/GAPDH and IL-1 1/GAPDH; CTR/GAPDH and OCN/GAPDH. Therefore, the ratios of IL-6/IL-11 and CTR/OCN were examined for any changes with age. Both IL-6/IL-11 (OA, r = - 0.12, p = NS; control, r = +0.15, p = NS) and CTR/OCN (OA, r = +0.04, p = NS; control, r = +0.26, p = NS) showed no dependency on age in either the OA or control group (Fig 6). however, the values for the two groups segregated such that, for the age range examined, the CTR/OCN mRNA values were higher in control individuals than in OA individuals.

OPG/GAPDH mRNA values have group means of 0.29±0.25 for OA compared to 0.21±0.08 for control samples (p=NS) (data not shown). When plotted versus age there was no dependence on age in either group, and no difference between groups with respect to expression versus age (data not shown). ODF/GAPDH mRNA values had group means of 0.54±0.51 for OA versus 0.73 ±0.47 for control samples (p-NS) (Figure 7). When plotted versus age a significant associated with age was found for ODF/GADPH for the control group ($p<0.02$). The association for the OA group did not achieve significance although segregation of the data for the two groups was found such that for each age examined ODF/GADPH values were lower in the OA for the control group (Figure 11). This difference between the groups resulted in the group means for the ratio of ODF/OPG being significantly different 1.93±0.87 for OA versus 3.41± 1.94 for control, $p<0.04$) (Figure 9)

RANK/GAPDH mRNA values have group means of 0.75 ±0.36 for OA were 0.33 versus 0.23 for control ($p<0.005$) (Figure 8). When plotted versus age neither the OA values nor the control values showed dependence on age (Figure 10), however the data were segregared such that at each age examined, the OA values were greater

than the control values, but with a divergence of OA and control values between the age of 40 to 50.

As can be seen from the above data we have determined the abundance of mRNA encoding IL-6 and IL-11 in OA and control bone. We have found by plotting the IL-6 and IL-11 values vs age that the OA and control values resolved into two distinct populations. Across the entire age range examined, spanning more than four decades of life, the OA values were lower than for age matched non-OA controls. Since this difference in IL-6 and IL-11 mRNA values was found to precede the difference between OA and control bone samples in terms of OCN expression we consider that it is predictive of the progression to severe OA. The difference in IL-6 and IL-11 levels may therefore be exploited as a predictive assay for OA.

EXAMPLE 3 - In vivo *measurement of serum marker levels*

The levels of known regulators of bone turnover regulators are assayed according to standard techniques (reviewed in Christiansen *et. al.*, Chapter 10, Metabolic Bone Disease, 1998, Academic Press). A blood or urine sample is taken from a patient having a family history of OA. The levels of selected marker(s) is assayed using the known techniques for that marker (see Christiansen *et. al., supra*). Data obtained is compared to data for a control group and data for an OA group and assessment is made based on the comparison. To avoid false negatives or positives the assessment is typically based on more than one marker. These markers are mostly measured by kit assay in routing laboratories, examples of suitable asays can be found in the following two references with are incorporated herein in their entirety. (Chiu *et al* (1999), Woitge *et al* (1999))

EXAMPLE 4 - *Predictive assessment of OA*

A patient aged 40 presents with a family history of OA. A sample of cancellous bone is taken by biopsy from the iliac crest and RNA is extracted as described in Example 1. mRNA is assayed by RT-PCR according to Example 1 using a panel of human specific oligonucleotide primers, and including in the assay for comparison RNA from a group of age matched normal control subjects (n>6), of known mRNA status for the mRNA species of interest. The following markers are measured and ratios calculated:

(i) IL-11/GAPDH

(ii) ODF/GAPDH

(iii) CTR/GAPDH
OCN/GAPDH

(iv) IL-6/GAPDH

(v) RANK/GAPDH

(vi) CTR/GAPDH

(vii) OCN/GAPDH

If the data fall outside of 2 standard deviations of the control data means values for IL-11 and two or more of the above parameters, the patient is considered at risk of suffering OA and preventative/therapeutic measures may be adopted.

Appropriate therapies that may be adopted could include episodic or continuous treatment, as required, with any of the agents presently known for the treatment of OA or with novel agents designed to inhibit progression of cancellous bone to the OA structure.. The patient should also be clinically monitored and parameters measured again in two years.

EXAMPLE 5 - *Predictive assessment of OA*

A patient of age 20 has a family history of OA. A sample of cancellous bone is taken by biopsy from the iliac crest and RNA is extracted as described in Example 1. mRNA is assayed by RT-PCR according to Example 1 using human specific oligonucleotide primers for IL-11. The result is compared with a group of age matched normal control subjects (n>6), of known IL-11 mRNA status. If the data fall outside of 2 standard deviations of the control data mean value, then the patient is considered at risk of suffering OA and should be followed with subsequent assays at

five year intervals. Follow up assays could include the more extensive screen procedure of Example 2.

Various features of the invention have been particularly shown and described in connection with the examples of the invention, however, it must be understood that these particular examples merely illustrate and that the invention is not limited thereto and can include various modifications falling within the spirit and scope of the invention.

REFERENCES

Chisaka and Capecchi (1991), *Nature*, **350**:473.

Chiu *et al* (1999), *J Bone Min Res* **14**: 609-615

Chomcynski and Sacchi (1987), *Anal Biochem* **162**: 156-159

Collins (1949) Osteoarthritis. *The Pathology of Articular and Spinal Diseases.* London, Edward Arnold & Co: 74-115

Dequeker *et al* (1983), *JAMA* **249**; 1448-1451

Dequeker *et al* (1993) *Arthritis & Rheumatism* **36**;1702-1708

Dequecker *et al* (1995) *Journal of Rheumatology* **22:1 Supplement 43**: 98-100

Dequecker *et al* (1997) *Microscopy Research and Technique* **37**: 358-371

Fazzalari *et al* (1998) *Bone* **22**: 381-388

Gaillard *et al* (1993) *Eur. J Immunol* **23**:820-824

Gevers and Dequeker (1987) *Collagen. Rel. Res.* **7**:435-442

Girasole *et al* (1994) *The Journal of Clinical Investigation* **93**:1516-1524

Hughes (1983) *British Journal of Hospital Medicine* **30**:234-242

Huges and Howells (1993) *Calcified Tissue Int* **53**:362-364

Hughes *et al* (1995) *J Bone Miner Res* **10**:533-544

Kishimoto *et al* (1995) *Blood* **86**:1243-1254

Manolagas (1995) *Bone* **17** :63S-67S

Manolagas and Jilka (1995) *N Eng J Med***332**:305-311

Marchuk *et al* (1997) *43rd Annual Meeting, Orthopaedic Research Society, February 9-13, 1997, San Fransisco, California:* 395

Miric *et. al.* (1992), *J Clin Endocrinol Metab*, **74**:509

Price *et. al.* (1981), *J Biol Chem*, **255**:2938-2942

Prockop *et. al.* (1979), *N Engl J Med*, **301**:12-23

Shanbhag *et al* (1995) *The Journal of Arthroplasty* **10**:498-506

Tamura *et al* (1993) *Cell Biology* **90**:11924-11928

Woitge *et al* (1999) *J Bone Min Res* **14**:792-801

CLAIMS

1. A method of predicting or diagnosing a skeletal disorder in an individual, said method including the steps of:

taking a sample of body tissue or body fluid,

measuring or estimating the level of at least one regulator or marker of bone remodelling in the sample, and

comparing the level to a standard to determine whether the level of the regulator or marker falls within a range indicative of a potential of the individual to progress to exhibit overt symptoms of the skeletal disorder.

2. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the sample includes at least a cellular component and the level of the regulator or marker is estimated by extracting mRNA from the cells of the sample and estimating the level of expression for the one or each of the regulators or markers of bone remodelling by measuring the quantity of mRNA specific for that regulator.

3. A method of predicting or diagnosing a skeletal disorder as in claim 2 wherein the sample is a bone sample.

4. A method of predicting or diagnosing a skeletal disorder as in claim 3 wherein the estimation of the level of regulator or marker is from cells isolated from regions other than the subchondral region of the bone.

5. A method of predicting or diagnosing a skeletal disorder as in claim 4 wherein the cells are isolated from bone from the iliac crest.

6. A method of predicting or diagnosing a skeletal disorder as in claim 3 wherein the level of one regulator or marker of bone remodelling is estimated.

7. A method of predicting or diagnosing a skeletal disorder as in claim 6 wherein the regulator or marker is selected from the group of regulators or markers associated with bone resorption comprising IL-1α, IL-1β, IL-6 and its soluble and cell membrane receptor, IL-11 and its receptor, IL-17 and its receptor, M-CSF, ODF, RANK, TRAIL and CTR, or the group of regulators or markers associated with bone

formation comprising BMP2, BMP6, BMP7, TGFβ1, TGFβ2, TGFβ3, IGFI, alkaline phosphatases, OCN, and fragments of type I collagen.

8. A method of predicting or diagnosing a skeletal disorder as in claim 7 wherein the regulator or marker is IL-11 or its receptor.

9. A method of predicting or diagnosing a skeletal disorder as in claim 3 wherein the levels of at least two regulators or markers of bone remodelling are estimated and an assessment is made on the basis of the combination of more than one score.

10. A method of predicting or diagnosing a skeletal disorder as in claim 9 wherein the regulators or markers are selected from the group of regulators or markers associated with bone resorption comprising IL-1α, IL-1β, IL-6 and its soluble and cell membrane receptor, IL-11 and its receptor, IL-17 and its receptor, M-CSF, ODF, RANK, TRAIL and CTR, or the group of inhibitors of bone resorption including IL-18 ad OPG or the group of regulators or markers associated with of bone formation comprising IL-18, OPG, BMP2, BMP6, BMP7, TGFβ1, TGFβ2, TGFβ3, IGFI, alkaline phosphatases, OCN, and fragments of type I collagen..

11. A method of predicting or diagnosing a skeletal disorder as in claim 10 wherein the regulator or marker is IL-11 or its receptor, and at least one of IL-6, RANK, CTR, OCN, OPG and ODF.

12. A method of predicting or diagnosing a skeletal disorder as in claim 10 wherein the ratio of a regulator or marker indicative of bone resorption compared to a regulator or marker indicative of bone formation is measured.

13. A method of predicting or diagnosing a skeletal disorder as in claim 12 wherein the ratio of the markers CTR/OCN mRNA is measured.

14. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the regulator is a growth factor.

15. A method of predicting or diagnosing a skeletal disorder as in claim 3 wherein the regulator is a localised bone autocrine or paracrine regulator or associated protein or receptor.

16. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the level of the regulator or marker is measured or estimated by assaying for activity *in vivo*.

17. A method of predicting or diagnosing a skeletal disorder as in claim 16 wherein the sample is a blood or urine sample.

18. A method of predicting or diagnosing a skeletal disorder as in claim 17 wherein the regulator or marker is selected from the group of regulators or markers indicative of bone resorption comprising IL-1α, IL-1β, IL-6 and its soluble and cell membrane receptor, IL-11 and its receptor, IL-17 and its receptor, M-CSF, ODF, RANK, TRAIL and CTR, or the group of regulators or markers indicative of bone formation comprising IL-18, OPG, BMP2, BMP6, BMP7, TGFß1, TGFß2, TGFß3, IGFI, alkaline phosphatases, OCN, and fragments of type I collagen.

19. A method of predicting or diagnosing a skeletal disorder as in claim 18 wherein the level of one regulator or marker of bone remodelling is measured or estimated.

20. A method of predicting or diagnosing a skeletal disorder as in claim 19 wherein the level of at least two regulators or markers of bone remodelling are measured or estimated and an assessment is made on the basis of the combination of more than one score.

21. A method of predicting or diagnosing a skeletal disorder as in claim 18 wherein the level of the regulator or marker is measured or estimated by assaying using monoclonal antibodies to the regulators or markers concerned.

22. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the disorder is osteoarthritis.

23. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the disorder is osteoporosis.

24. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the method is used for mammals.

25. A method of predicting or diagnosing a skeletal disorder as in claim 24 wherein the humans.

26. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the standard is ascertained by referring to a table or graph.

27. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the standard is ascertained by referring to an experimental control.

AMENDED CLAIMS

[received by the International Bureau on 2 December 1999 (02.12.99);
original claims 1-27 replaced by amended claims 1-26 (4 pages)]

1. A method of predicting or diagnosing a skeletal disorder in an individual, said method including the steps of:

taking a sample of body tissue or body fluid, said sample including at least a cellular component,

measuring or estimating the level of at least one regulator or marker of bone remodelling in the sample by extracting mRNA from the cells of the sample and estimating the level of expression for the one or each of the regulators or markers of bone remodelling by measuring the quantity of mRNA specific for that regulator or marker, and

comparing the level to a standard to determine whether the level of the regulator or marker falls within a range indicative of a potential of the individual to progress to exhibit overt symptoms of the skeletal disorder.

2. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the sample is a bone sample.

3. A method of predicting or diagnosing a skeletal disorder as in claim 2 wherein the estimation of the level of regulator or marker is from cells isolated from regions other than the subchondral region of the bone.

4. A method of predicting or diagnosing a skeletal disorder as in claim 3 wherein the cells are isolated from bone from the iliac crest.

5. A method of predicting or diagnosing a skeletal disorder as in claim 2 wherein the level of one regulator or marker of bone remodelling is estimated.

6. A method of predicting or diagnosing a skeletal disorder as in claim 5 wherein the regulator or marker is selected from the group of regulators or markers associated with bone resorption comprising IL-1α, IL-1β, IL-6 and its soluble and cell membrane receptor, IL-11 and its receptor, IL-17 and its receptor, M-CSF, ODF, RANK, TRAIL and CTR, or the group of regulators or markers associated with bone formation comprising IL-18, OPG, BMP2, BMP6, BMP7, TGFβ1, TGFβ2, TGFβ3, IGFI, alkaline phosphatases, OCN, and fragments of type I collagen.

7. A method of predicting or diagnosing a skeletal disorder as in claim 6 wherein the regulator or marker is IL-11 or its receptor.

8. A method of predicting or diagnosing a skeletal disorder as in claim 2 wherein the levels of at least two regulators or markers of bone remodelling are estimated and an assessment is made on the basis of the combination of more than one score.

9. A method of predicting or diagnosing a skeletal disorder as in claim 8 wherein the regulators or markers are selected from the group of regulators or markers associated with bone resorption comprising IL-1α, IL-1β, IL-6 and its soluble and cell membrane receptor, IL-11 and its receptor, IL-17 and its receptor, M-CSF, ODF, RANK, TRAIL and CTR, or the group of inhibitors of bone resorption including IL-18 and OPG or the group of regulators or markers associated with of bone formation comprising IL-18, OPG, BMP2, BMP6, BMP7, TGFβ1, TGFβ2, TGFβ3, IGFI, alkaline phosphatases, OCN, and fragments of type I collagen.

10. A method of predicting or diagnosing a skeletal disorder as in claim 9 wherein the regulator or marker is IL-11 or its receptor, and at least one of IL-6, RANK, CTR, OCN, OPG and ODF.

11. A method of predicting or diagnosing a skeletal disorder as in claim 9 wherein the ratio of a regulator or marker indicative of bone resorption compared to a regulator or marker indicative of bone formation is measured.

12. A method of predicting or diagnosing a skeletal disorder as in claim 11 wherein the ratio of the markers CTR/OCN mRNA is measured.

13. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the regulator is a growth factor.

14. A method of predicting or diagnosing a skeletal disorder as in claim 2 wherein the regulator is a localised bone autocrine or paracrine regulator or associated protein or receptor.

15. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the disorder is osteoarthritis.

16. A method of predicting or diagnosing a skeletal disorder as in claim 1 wherein the disorder is osteoporosis.

17. A method of predicting or diagnosing osteoarthritis, said method including the steps of:

taking a sample of body tissue or body fluid,

measuring or estimating the level of at least one regulator or marker of bone remodelling in the sample by assaying for levels of the regulator or marker *in vivo*, and

comparing the level to a standard to determine whether the level of the regulator or marker falls within a range indicative of a potential of the individual to progress to exhibit overt symptoms of osteoarthritis.

18. A method of predicting or diagnosing osteoarthritis as in claim 17 wherein the sample is a blood or urine sample.

19. A method of predicting or diagnosing osteoarthritis as in claim 18 wherein the regulator or marker is selected from the group of regulators or markers indicative of bone resorption comprising IL-1α, IL-1β, IL-6 and its soluble and cell membrane receptor, IL-11 and its receptor, IL-17 and its receptor, M-CSF, ODF, RANK, TRAIL and CTR, or the group of regulators or markers indicative of bone formation comprising IL-18, OPG, BMP2, BMP6, BMP7, TGFß1, TGFß2, TGFß3, IGFI, alkaline phosphatases, OCN, and fragments of type I collagen.

20. A method of predicting or diagnosing osteoarthritis as in claim 19 wherein the level of one regulator or marker of bone remodelling is measured or estimated.

21. A method of predicting or diagnosing osteoarthritis as in claim 19 wherein the level of at least two regulators or markers of bone remodelling are measured or estimated and an assessment is made on the basis of the combination of more than one score.

22. A method of predicting or diagnosing osteoarthritis as in claim 20 wherein the level of the regulator or marker is measured or estimated by assaying using monoclonal antibodies to the regulators or markers concerned.

23. A method of predicting or diagnosing osteoarthritis as in claim 17 wherein the method is used for mammals.

24. A method of predicting or diagnosing osteoarthritis as in claim 23 wherein the method is used for humans.

25. A method of predicting or diagnosing osteoarthritis as in claim 17 wherein the standard is ascertained by referring to a table or graph.

26. A method of predicting or diagnosing osteoarthritis as in claim 17 wherein the standard is ascertained by referring to an experimental control.

STATEMENT UNDER ARTICLE 19

1. Claim 1 has been amended and takes in part of claim 2.
2. Claim 2 has been cancelled.
3. Claims 3 to 15 inclusive have been renumbered 2 to 14 inclusive and the appendences have been amended accordingly.
4. New Claim 15 has been inserted.
5. New Claim 16 has been inserted.
6. Claims 16 to 21 inclusive have been renumbered 17 to 22 inclusive and amended.
7. Claims 22 and 23 have been cancelled.
8. Claims 24 to 27 inclusive have been renumbered 23 to 26 inclusive and amended.

For your convenience retyped pages 19 to 22 containing these amendments is forwarded together with a redlined copy of the Claims showing where the abovementioned amendments have been made.

TABLE 1. PCR PRIMERS AND PREDICTED PCR PRODUCT SIZES

GENE		PRIMER SEQUENCE (5'-3')	PCR PRODUCT SIZE (bp)
IL-6	Sense	ATGAACTCCTTCTCCACAAG	544
	Antisense	GTGCCTGCAGCTTCGTCAGCA	
IL-11	Sense	GACATGAACTGTGTTTGCCGCCTGG	289
	Antisense	TTGTCAGCACACCTGGGAGCTGTAG	
OCN	Sense	ATGAGAGCCCTCACACTCCTCG	255
	Antisense	GTCAGCCAACTCGTCACAGTCC	
CTR	Sense	GCAATGCTTTCACTCCTGAGAAACT	732/780
	Antisense	AGTGCATCACGTAATCATATATC	
OPG	Sense	TGCTGTTCCTACAAAGTTTACG	435
	Antisense	CTTTGAGTGCTTTAGTGCGTG	
ODF	Sense	AATAGAATATCAGAAGATGGCACTC	668
	Antisense	TAAGGAGGGGTTGGAGACCTCG	
RANK	Sense	CCTACGCACAAGGCGAAGATGC	704
	Antisense	CGTAGACCACGATGATGTCGCC	
GAPDH	Sense	CATGGAGAAGGCTGGGGCTC	415
	Antisense	CACTGACACGTTGGCAGTGG	

TABLE 2

	OA		CONTROL	
	Female (n = 8)	Male (n = 8)	Female (n = 7)	Male (n = 7)
IL-6/GAPDH	0.11 ± 0.04	0.13 ± 0.08	0.23 ± 0.16	0.15 ± 0.04
IL-11/GAPDH	0.12 ± 0.04	0.09 ± 0.02*	0.28 ± 0.12	0.18 ± 0.05
CTR/GAPDH	0.65 ± 0.32	0.55 ± 0.30	0.46 ± 0.23	1.39 ± 0.88*
OCN/GAPDH	1.36 ± 0.57	1.14 ± 0.51	0.37 ± 0.11	0.32 ± 0.10

* $p < 0.05$

FIGURE 1

OA

CONTROL

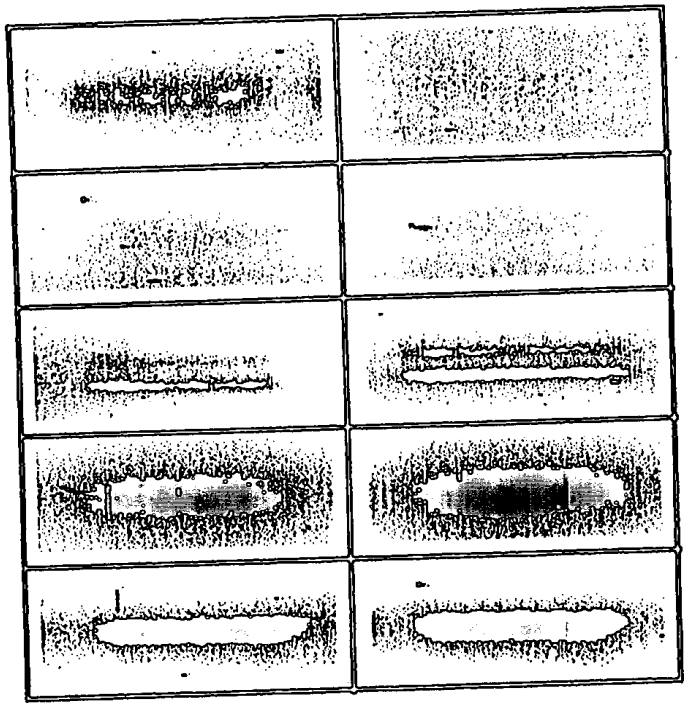
IL-6

IL-11

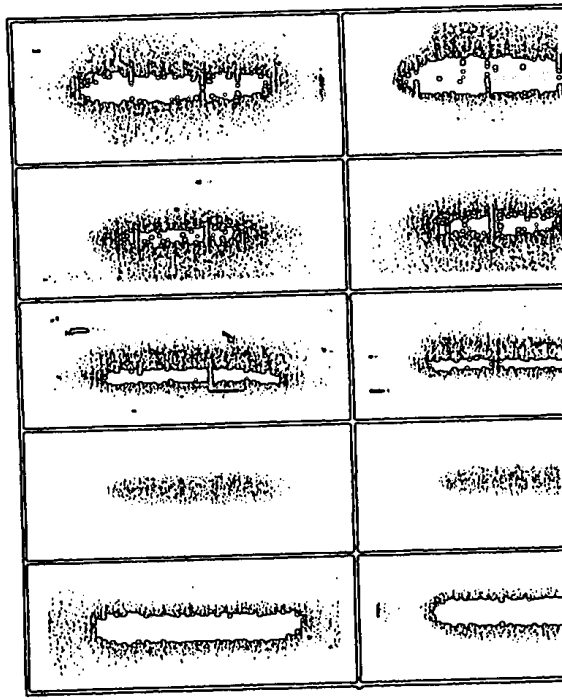
CTR

OCN

GAPDH



M 50 F 80



M 44 F 7

FIGURE 2A

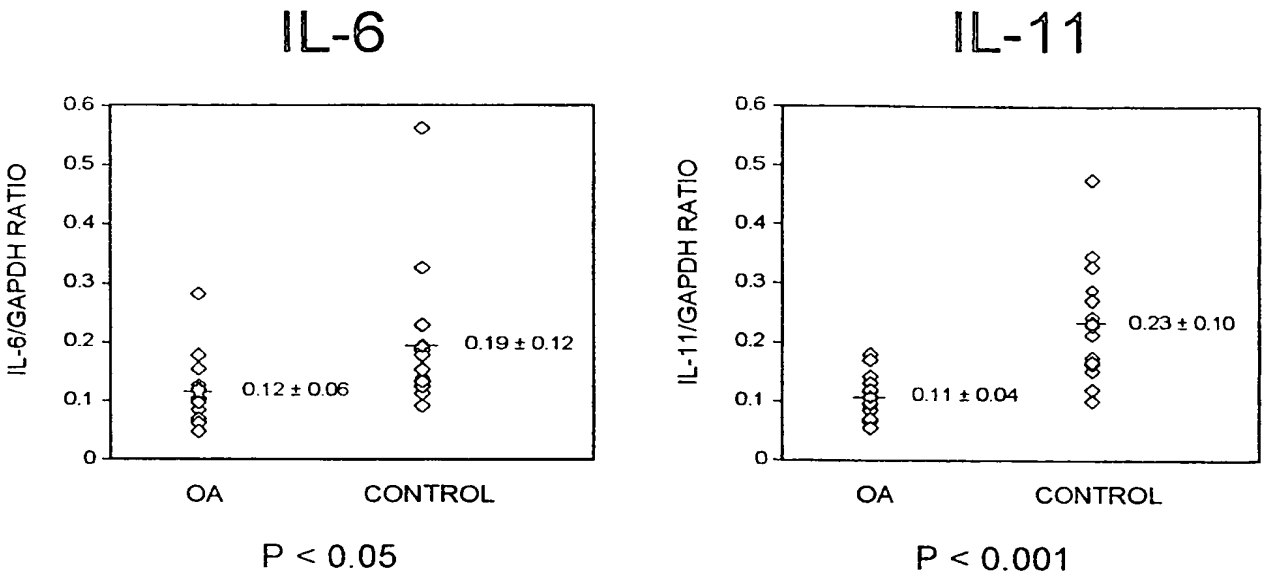


FIGURE 2B

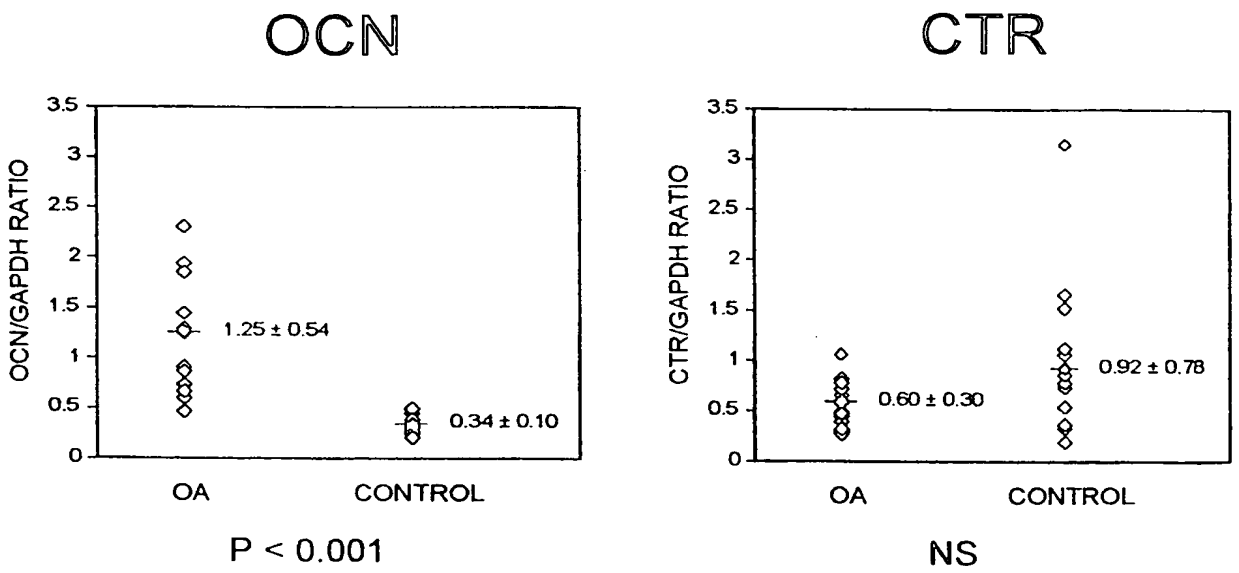


FIGURE 3

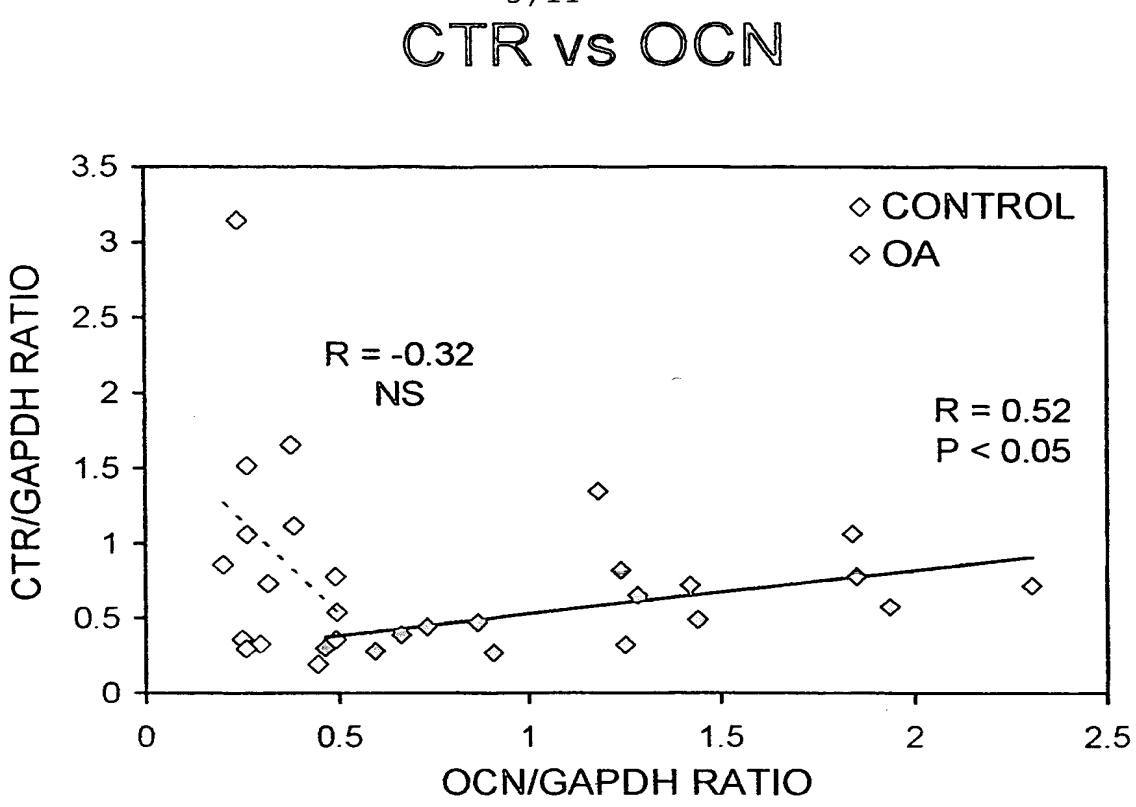


FIGURE 4

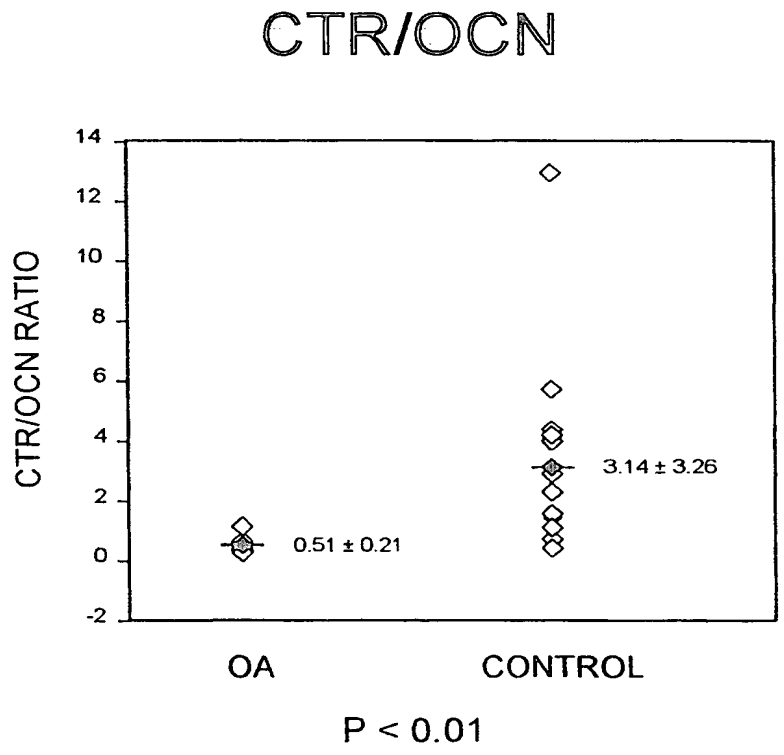


FIGURE 5A

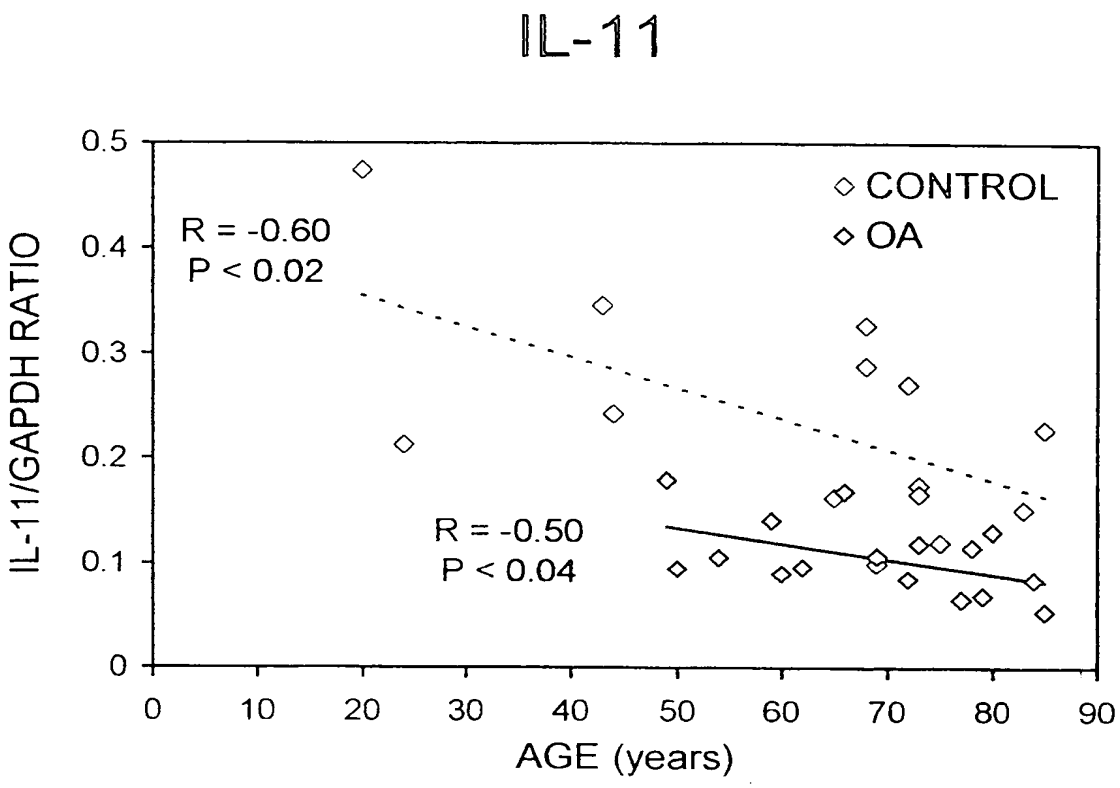


FIGURE 5B

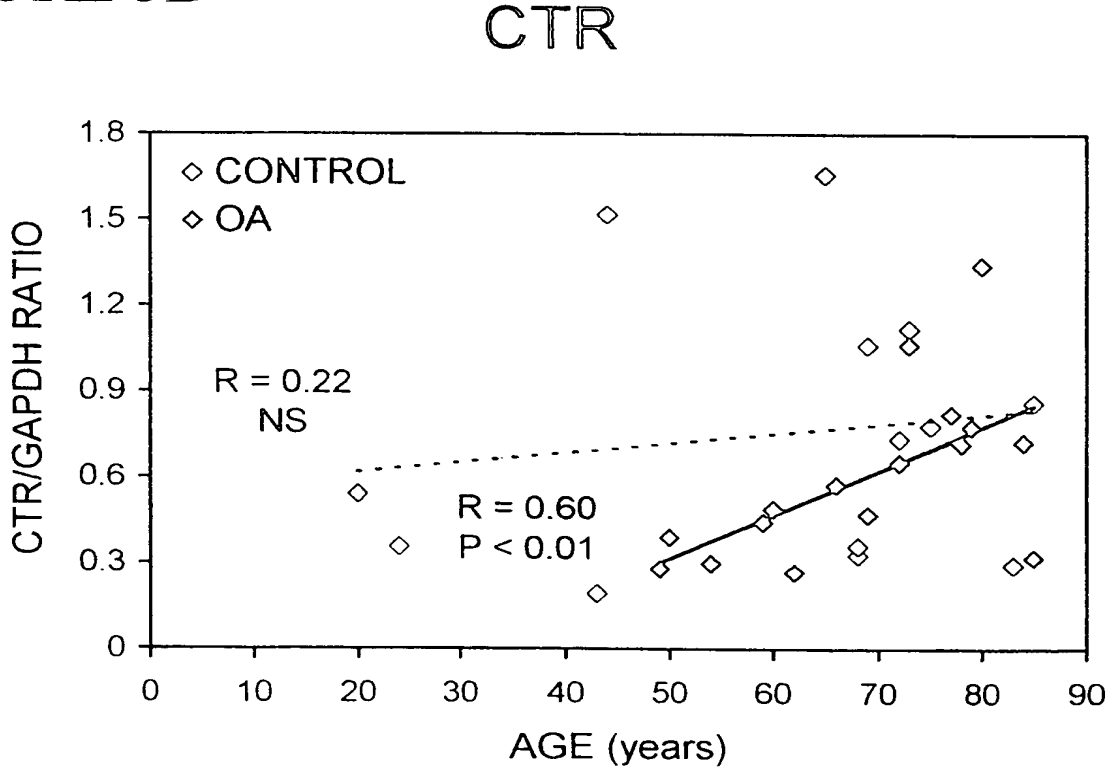


FIGURE 5C

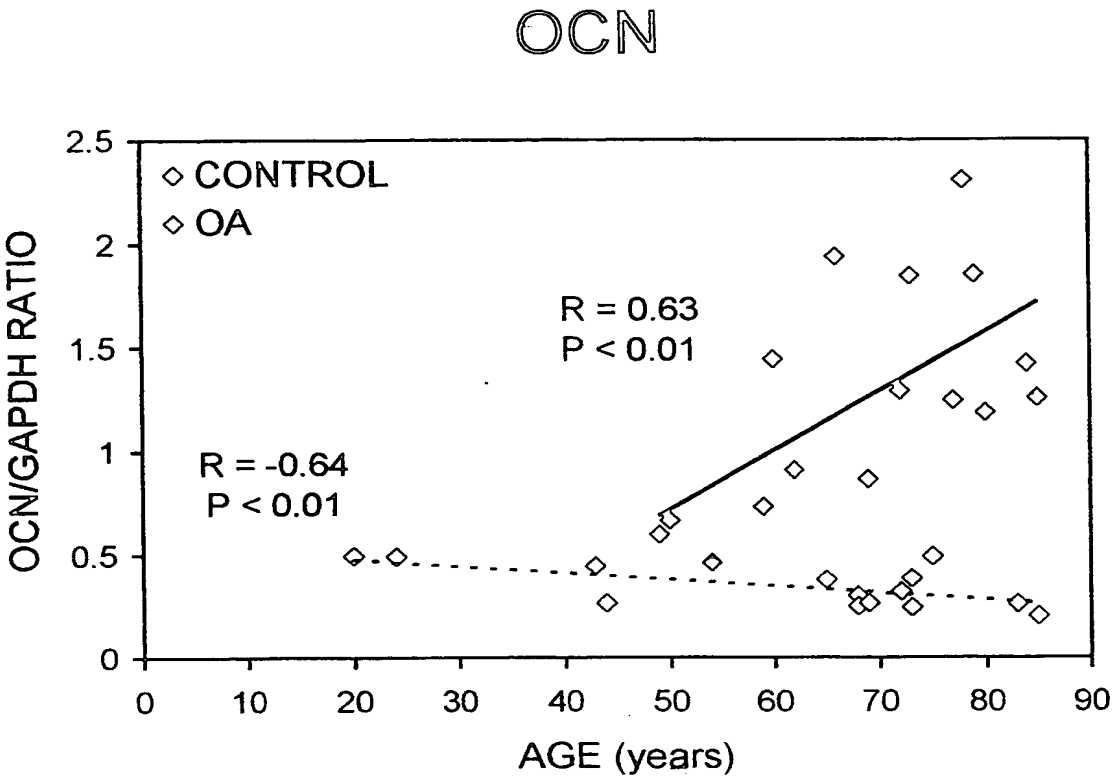

OCN
CONTROL
OA
R = 0.63
P < 0.01
R = -0.64
P < 0.01
OCN/GAPDH RATIO
AGE (years)
0
0.5
1
1.5
2
2.5
0
10
20
30
40
50
60
70
80
90

FIGURE 6

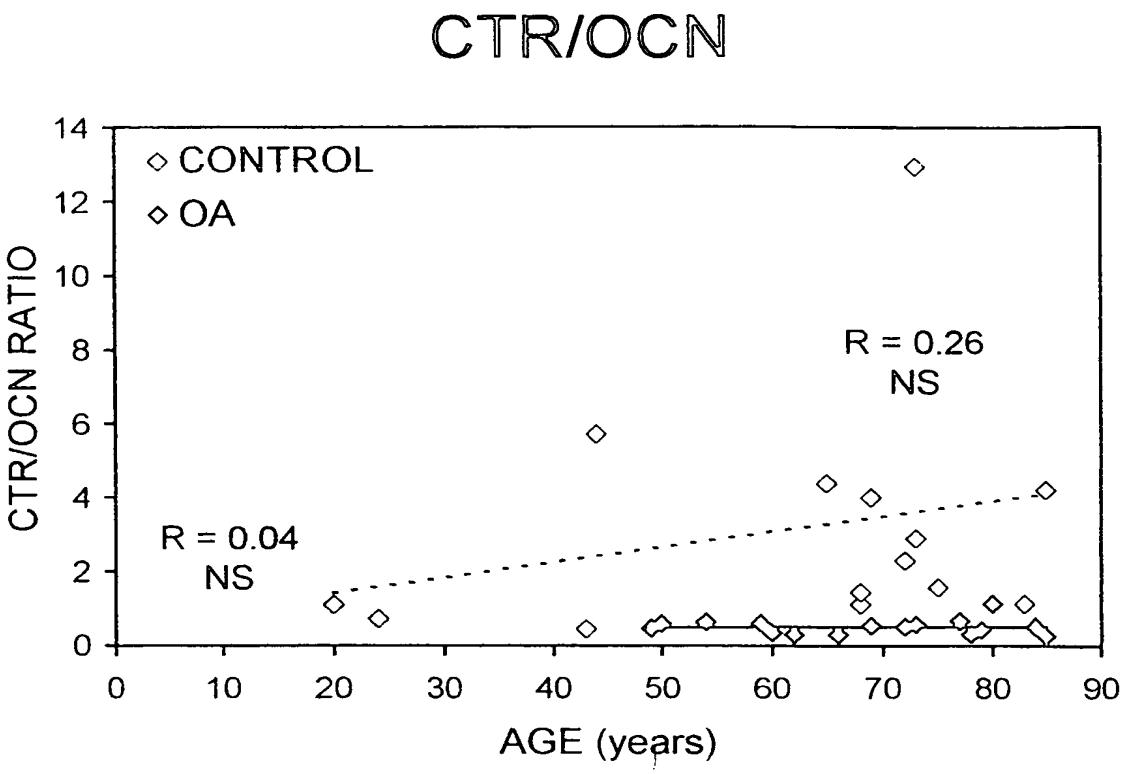


FIGURE 7

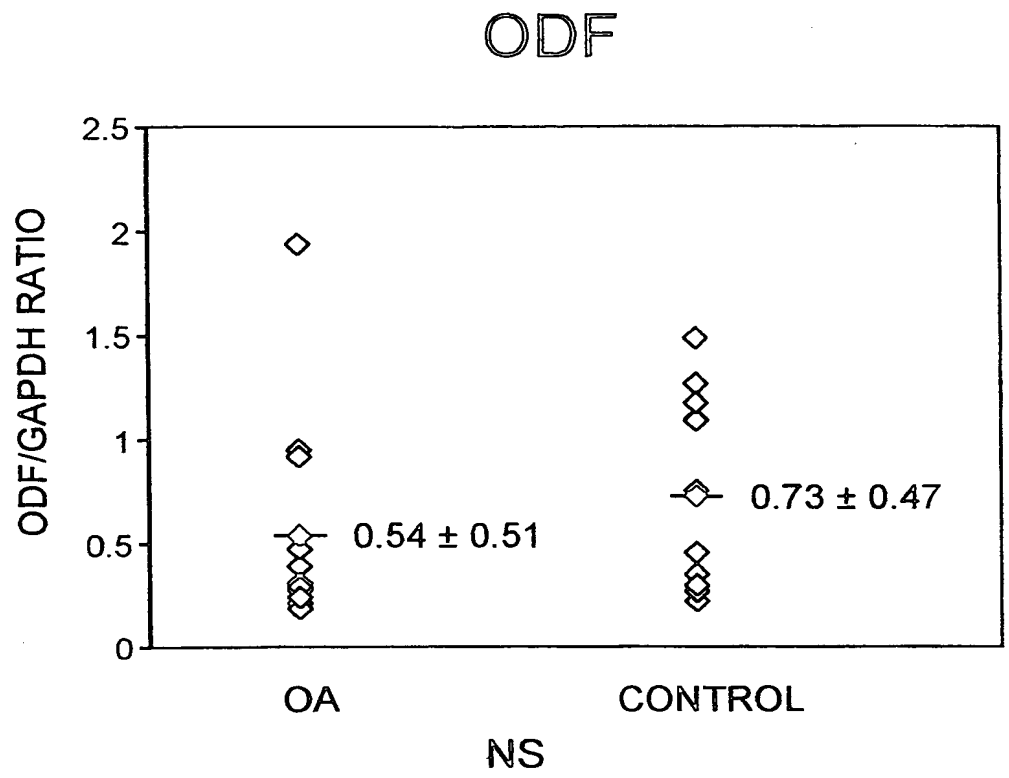


FIGURE 8

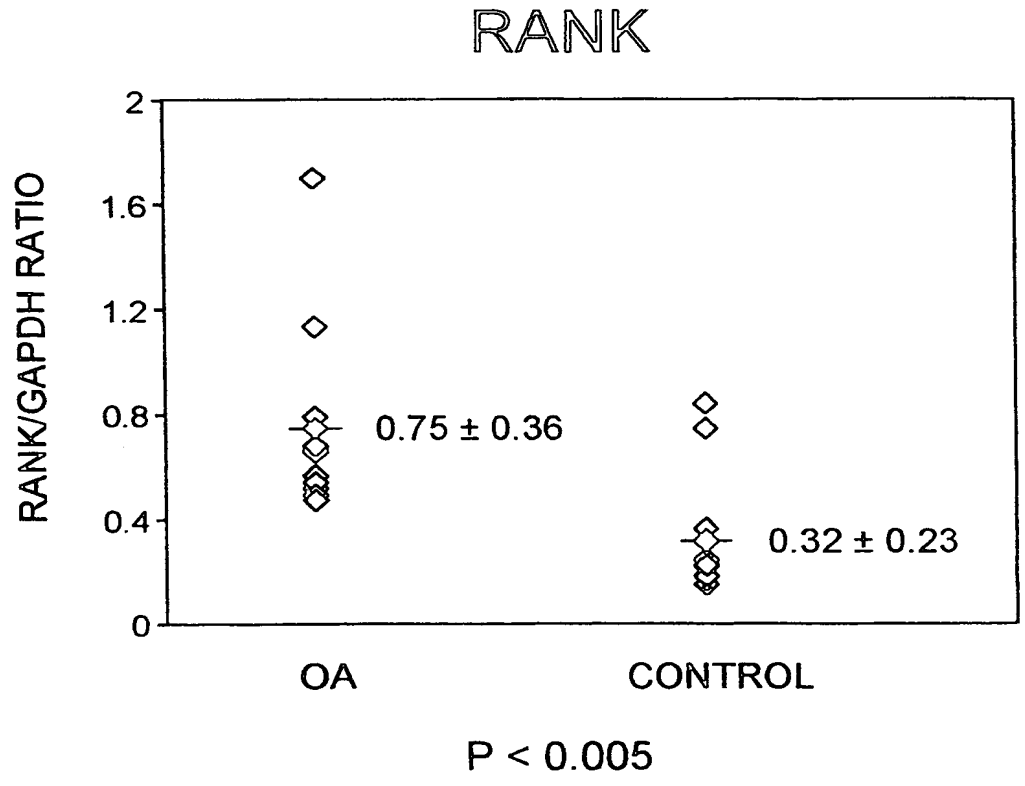
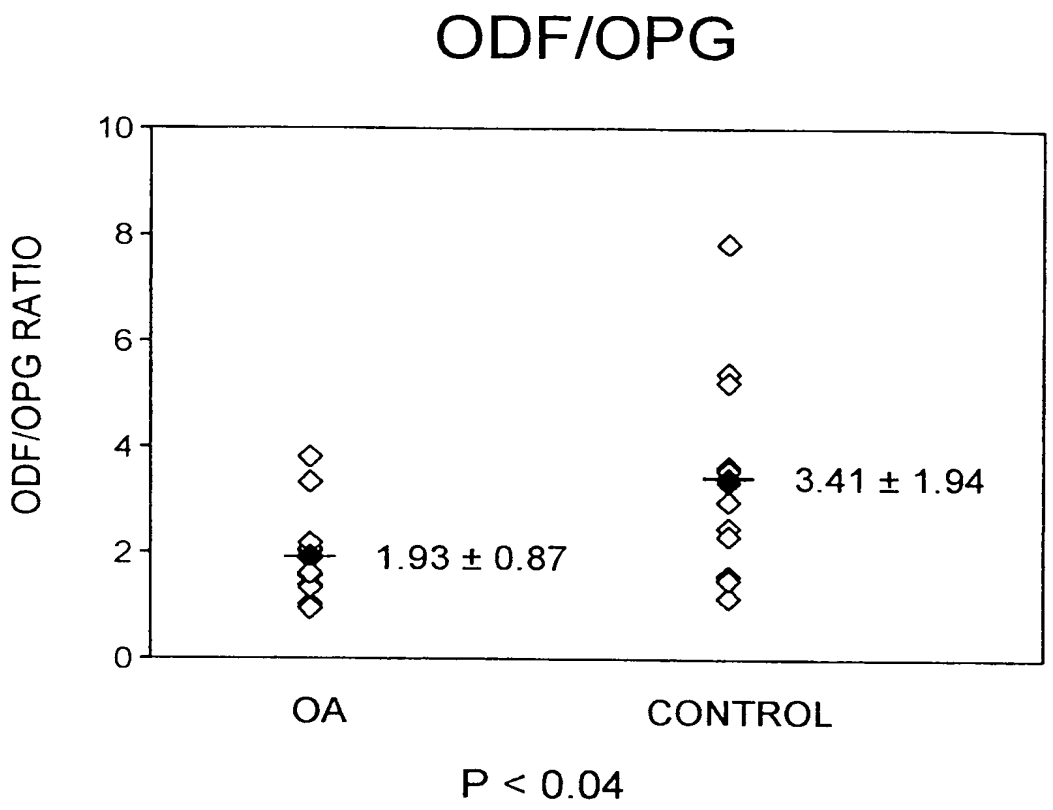


FIGURE 9



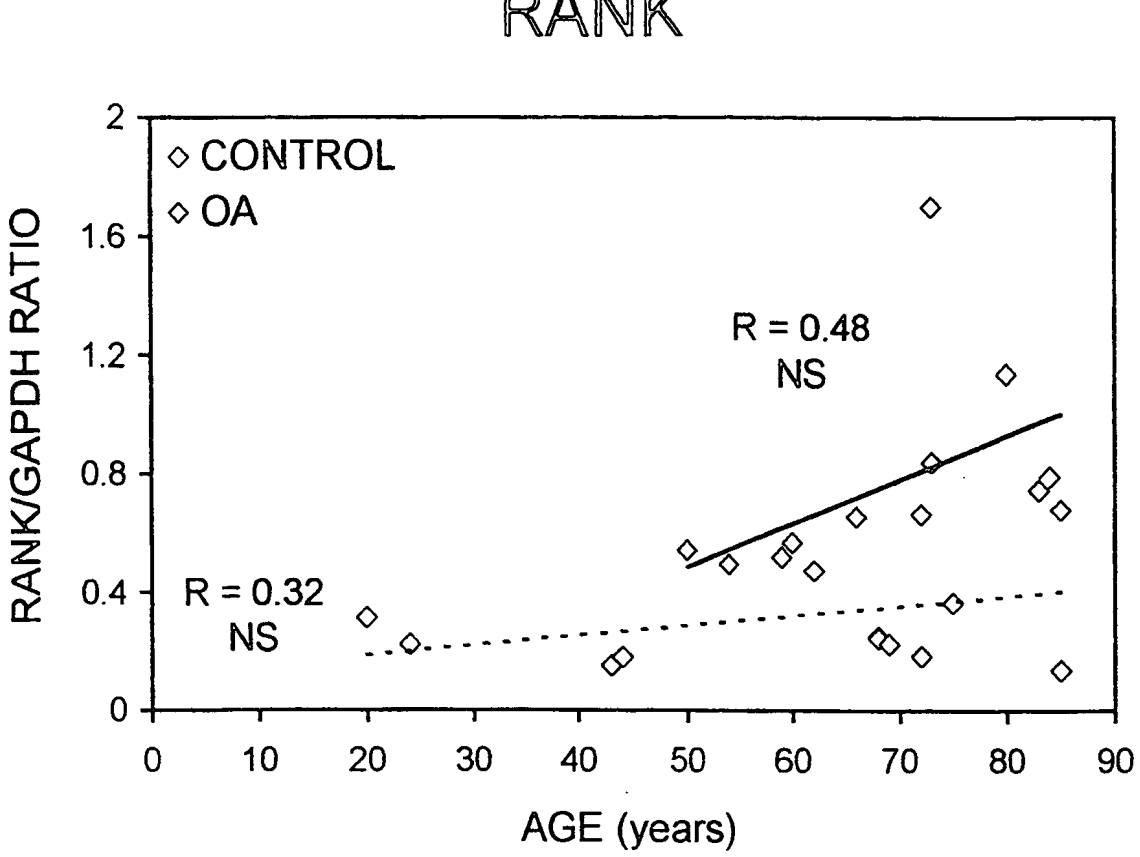
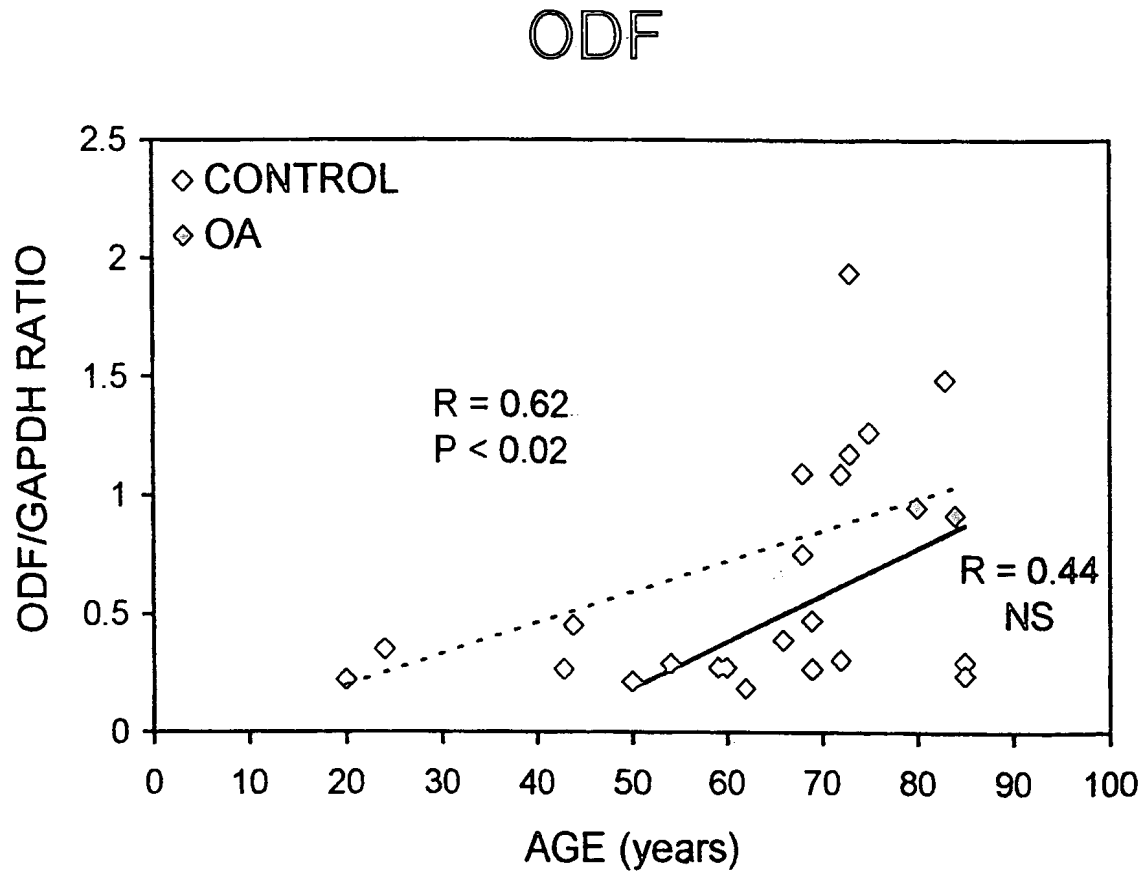


FIGURE 11



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00697

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 33/577 C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: G01N 33/- C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC AS ABOVE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT JAPIO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/07032 A (METRA BIOSYSTEMS, INC.) 19 February 1998 Pages 3-5, Examples 2-7, Claims 1-17	1,3,4,6,7,22-27
X	WO 93/15107 A (Baylink et al) 5 August 1993 Page 3, Examples 1-3	1,6,7,23-27
X	WO 97/38135 A (MEDICAL SCIENCE SYSTEMS, INC.) 16 October 1997 Claims 1-7	1,6,7,23-27

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 1999	07 OCT 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **N.L. KING** Telephone No.: (02) 6283 2150

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00697

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/43446 A (GEMINI INTERNATIONAL HOLDINGS LIMITED) 20 November 1997 Claims 1-29	1,6,7,23-27
X	EP 557663 A (CENTRE DE RECHERCHES EN RHUMATOLOGIE) 1 September 1993 Claims 1-23	1,6,7,23-27

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/00697

Patent Document Cited in Search Report		Patent Family Member					
WO	9807032	AU	41520/97				
WO	9315107	EP	631586	US	5599679		
WO	9738135	AU	26077/97	CA	2226223	EP	832298
		US	5698399	ZA	9702909		
WO	9743446	CA	2254901	EP	912759	GB	9610281
		AU	29041/97				
EP	557663	JP	6078788				
							END OF ANNEX